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Characterization of Antisense RNA-Mediated Inhibition of SIV Replication

Frank Y.-T. Tung and Man-Hwa Tung

Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pennsylvania

Antisense RNA-mediated inhibition of HIV showed mixed success in previous experiments. In order to elucidate the parameters influencing the efficacy of an antisense RNA approach, retroviral vectors encoding 4.5 kb, 3.5 kb, or 2.5 kb antisense RNA of the *gag-pol* region of SIV (simian immunodeficiency virus) were constructed and used to transduce a CD4-positive CEM174 cell line. The growth rate of transduced cells was measured, and results showed that antisense RNAs have no detrimental effect on cell growth. Similar levels of antisense RNA expression were observed in all transduced cells by Northern analysis. The transduced cells were challenged with uncloned SIVmac239 at a m.o.i. (multiplicity of infection) of 1.0, 0.1, or 0.01. At a m.o.i. of 1.0 or 0.1, no significant inhibition of viral replication was observed in any antisense construct transduced cells up to 9 days postinfection. At a lower m.o.i. (0.01), viral replication was effectively inhibited in 3.5 kb antisense transduced cells as compared to 4.5 kb and 2.5 kb antisense transduced cells at 15 days postinfection. These data suggest that the size of antisense RNA and the challenge dose play a significant role in achieving effective inhibition.

KEY WORDS: antisense RNA, SIV, gene therapy, AIDS

INTRODUCTION

In a previous study of an SIV model, it was demonstrated that 3.5 Kb (envelope gene region) antisense RNAs can inhibit effectively SIV replication in a lymphocyte line (Hut-78) and 5.6 Kb antisense RNAs (*gag-pol* region) can inhibit both SIV and HIV replication [Tung and Daniel, 1993; Tung, 1994]. In this report, we characterize further the effect of antisense RNA target size and infectious dose on inhibition of viral replication. The goal is to determine the optimal size of effective antisense RNA in inhibiting viral replication in a condition similar to natural infection (e.g., low viral titer).

Early experiments from other investigators also showed that small antisense RNA (<1 Kb) achieved only partial or transient inhibition of HIV replication [Joshi et al., 1991; Lori et al., 1994; Rhodes and James, 1990; Sczakiel and Pawlita, 1991]. Viral replication broke through 2–3 weeks after infection in the antisense expressing cells. In those studies, the assay conditions were not ideal, because either clonal selected cells or cells transfected with large quantities of antisense constructs were used. The optimal goal of gene therapy for AIDS is the protection of uninfected hematopoietic stem cells. The lymphocytes derived from transduced stem cells will carry single copies of an antisense gene in most cases. In this study, an amphotropic virus was used as the gene transfer vector for antisense gene transduction. The full-length viral RNA is the target sequence to eliminate other non-antisense effects, i.e., RNA decoy. Retroviral vectors encoding 4.5 Kb, 3.5 Kb, and 2.5 Kb antisense RNA of the *gag-pol* genes were constructed and used to transduce the CD4 positive lymphocyte cell line, CEM174. The transduced cells were challenged with an uncloned SIV macaque isolate at different m.o.i.s. The purpose of this experiment was to demonstrate the importance of the size of antisense RNA for effective inhibition and the role of viral dose for challenging.

MATERIALS AND METHODS

Virus

SIV macaque isolate 239 was grown in the CEM174 cell [Salter et al., 1985] as described previously [Tung and Daniel, 1993]. The viral titer was determined by infection on CEM174 cells with two-fold series of dilutions in 24-well plates. The culture was passaged every 3 days at 1:3 dilution. The positive infectivity was identified by syncytium formation after 2–3 weeks of incubation at 37°C in a CO_2 incubator.

Construction of Retroviral Vectors and Producer Lines

DNA fragments corresponding to 4.5 kb, 3.5 kb, and 2.5 kb of *gag-pol* genes were PCR amplified from plas-

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Address reprint requests to Frank Y.-T. Tung, Department of Infectious Diseases and Microbiology, 130 DeSoto Street, Room 439, Pittsburgh PA 15261.

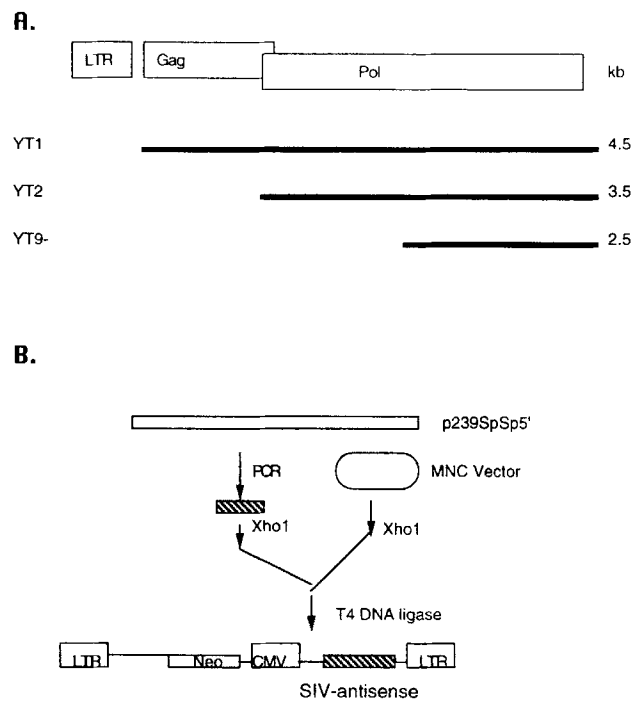


Fig. 1. Retroviral constructs. The positions of antisense genes corresponding to the SIV genome and the size are indicated **(A)**. Structure of expression vectors and construction scheme **(B)**.

mid p239SpSp5' [Regier and Desrosiers, 1990] with specific primer pairs and subcloned into murine retroviral vector pMNC (Fig. 1). There is an Xhol restriction site built in in each primer. The clones with the insert in antisense orientation were identified by restriction mapping. Primer pair I (FT22 plus FT24), II (FT23 plus FT24), and III (FT29 plus FT24) were used for amplifying 4.5 kb, 3.5 kb, and 2.5 kb fragments, respectively. The primer sequences were as follows. FT22: 5'ACGCTCGAGTGCTCCTATAAAGGCGCG, FT23: 5'CCACTCGAGCCATTTCAGAGCTATGTAGA, FT24: 5' TTTCTCGAGGCTATGCCACCTCTCTAG and FT29: 5' CATCTCGAGAAATCTGTGAAAAGAT. The PCR conditions were 30 cycles of each, consisting of 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes. The high titer retroviral producer lines were generated as previously described [Tung and Daniel, 1993].

Transduction of CEM174 Cells and Infection With SIVmac

Stable transduced CEM174 cells were established by co-culture with the producer line. The transduced cells were infected with SIVmac at m.o.i.'s of 1.0, 0.1, and 0.01. Culture suspension (2½ ml) were removed at different days of postinfection for reverse transcriptase (RT) assay. Complete medium (2½ ml) were added into each cell culture after each removal. Viral replication was monitored by RT assay as previously described [Tung and Daniel, 1993].

Northern Analysis

Total RNAs were extracted from transduced cells by the guanidium thiocynate method, then hybridized with a 2.5 kb ^{32}P-labeled *pol* probe prepared by PCR with primers FT29 and FT24.

PCR Analysis of Infected Cells

Total DNA was extracted from infected cells at 9 and 13 days postinfection by the proteinase K/phenol method. DNA (0.5 μg) was used to co-amplify SIV *env* (2290 bp) and *pol* (923 bp) genes. One ng DNA was used to co-amplify *env* and *Neo*^{r} gene (794 bp). The PCR conditions were the same as above, but 35 cycles were used instead. The primers used for *pol* gene were FT23 and FT28 (5'GAGACATCCCCAGAGCTGTTAG). The other primer sequences for PCR were the same as described [Tung and Daniel, 1993].

Immunoblotting of Infected Cells

Total cell lysates were harvested from infected cells at 15 days postinfection and subjected to 10% SDS-PAGE analysis. After proteins were resolved on the gel and transferred onto nitrocellulose membranes, the blot was reacted with anti-SIV antibody from seropositive monkeys. The positive bands were detected by peroxidase cojugated mouse anti-monkey antibody (Sigma Chemicals, St. Louis, MO) with a chemiluminescent agent (Amersham, Arlington Heights, IL).

RESULTS

Construction of Amphotropic Retroviral Vectors

The PCR amplified DNAs were subcloned into the pMNC vector to construct pYT1, pYT2, and pTY9.1-encoding 4.5 kb, 3.5 kb, and 2.5 kb antisense sequences of the *gag-pol* region, respectively (Fig. 1). The corresponding virus producer lines were generated with a titer of 10^5–10^6 cfu/ml for each construct.

Transduction of CEM174 Cells and Growth Rate of Transduced Cells

CEM174 cells were cocultured with the amphotropic producer line for 16–20 hours, then selected with 0.5 mg/ml G418 (active form). The growth rate of transduced cells was determined by measuring the doubling time. The results showed the same growth rate (22 hr) for vector and antisense transduced cells (data not shown).

Northern Analysis of Transduced CEM174 Cells

Total RNA was extracted from transduced cells that were selected with G418 (0.5 mg/ml) for at least 2 weeks, then grown in the absence of selection. RNA (10 μg) was probed with the 2.5 kb *pol* probe. Two RNA bands transcribed either from LTR (upper bands; 6.5 kb, 5.5 kb, 4.5 kb in YT1, YT2, and YT9.1-transduced cells, respectively) or internal CMV promoters (lower bands; 4.5 kb, 3.5 kb, 2.5 kb in YT1, YT2, and YT9.1-transduced

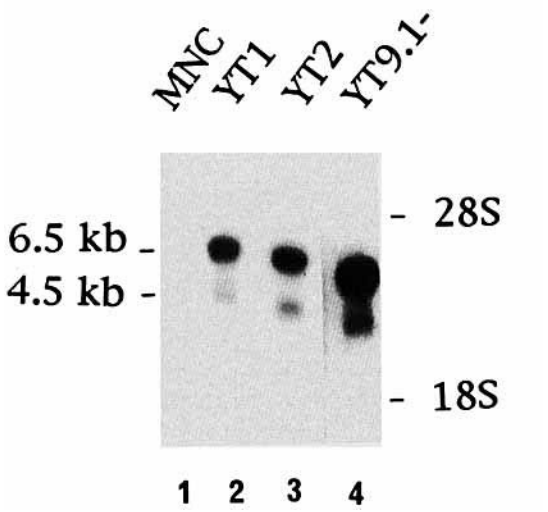


Fig. 2. Northern analysis. Total RNA extracted from transduced cells were probed with *pol* probe. MNC was vector transduced cells as control. One Kb ladder (Gibco/BRL) was used as migration indicator. The positions of ribosomal RNAs (28S and 18S) are also indicated.

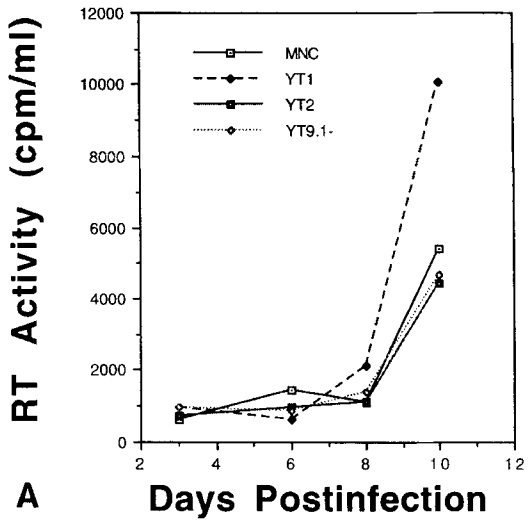
cells, respectively) were identified (Fig. 2). The upstream LTR promoter had a dominant effect. However, the expression level of antisense RNA was similar in each sample of the transduced cells.

Inhibition of SIVmac in Transduced CEM174 Cells

The pools of transduced cells were challenged with uncloned SIVmac239 at m.o.i. of 1.0, 0.1, or 0.01. At a m.o.i of 1.0 or 0.1, no significant inhibition of viral replication was observed in any antisense construct transduced cells up to 9 days postinfection. At a lower m.o.i. (0.01), viral replication was effectively inhibited in 3.5 kb antisense transduced cells as compared to 4.5 kb and 2.5 kb antisense transduced cells at 15 days postinfection (Fig. 3). The viable cells were counted at 15 days postinfection. The YT2 transduced cells had five times more viable cells than the MNC vector transduced cells (Table I). The RT assay was terminated after 15 days postinfection due to significant cell death in unprotected cell populations.

Analysis of SIV Proteins and Provirus in Antisense Transduced Cells

One mg of total cell lysate from infected cells (0.01 moi) was subjected to immunoblotting with anti-SIV antibody. Little or no SIV protein was detected in YT2 transduced cells as compared to other transduced cells at 15 days postinfection (Fig. 4). To determine the relative abundance of provirus in the infected cells (0.01 moi), 0.5 μg of total DNA was co-amplified with *env* and *pol* genes. The results indicated that all the cells were infected (Fig. 5A). However, SIV sequence was not detected in YT2 and YT9.1-transduced cells when 1 ng DNA was used for PCR at 9 days postinfection (Fig. 5B). The results indicated that the relative abundance of SIV is



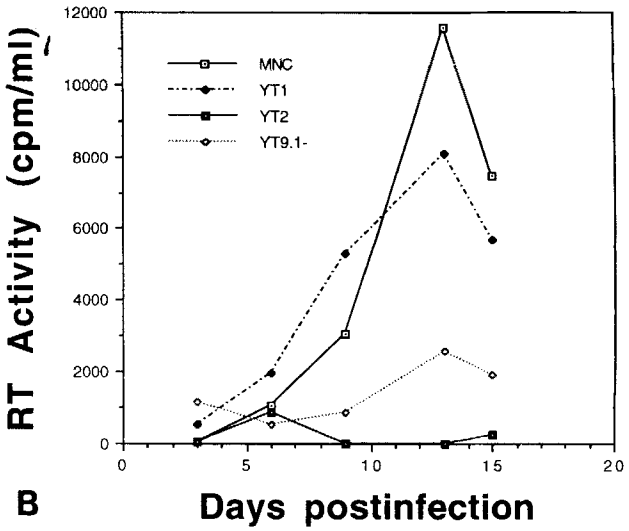


Fig. 3. RT assay of antisense transduced CEM174 cells. One million cells were infected with uncloned SIVmac 239 at m.o.i. of 0.1 **(A)** or 0.01 (10^5 or 10^4 $TCID_{50}$) **(B)**. Data shown represent means of four experiments.

TABLE I. Absolute Number of Viable Cells (per μl of total of 5 ml) Infected With SIVmac at m.o.i. of 0.01 at 15 Days Postinfection

Cell	MNC	YT1	YT2	YT9.1–
no.	30	32	131	99

lower in YT2 and YT9.1-transduced cells. These results suggest that the antisense RNA cannot completely prevent provirus integration and may interfere with viral gene expression at a posttranscription step.

DISCUSSION

The use of antisense RNA had been explored as a possible means to inhibit gene expression with different

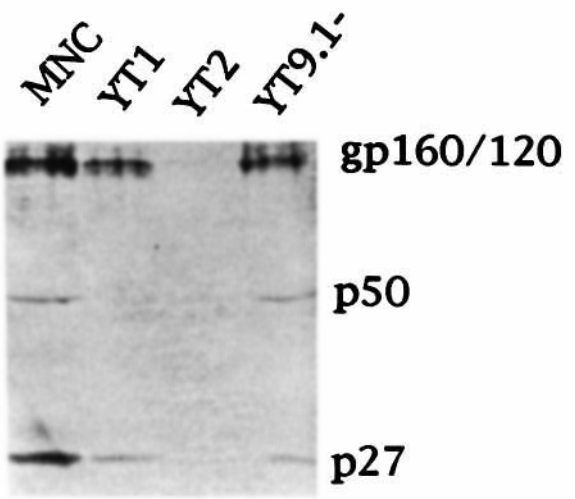


Fig. 4. Immunoblotting of SIVmac infected cells. Total cell lysates were collected from infected cells (0.01 moi) at 15 days postinfection and reacted with anti-SIV antibody. Three major SIV proteins, gp160/120, p50, and p27 were detected in vector (MNC), YT1, and YT9.1-transduced cells.

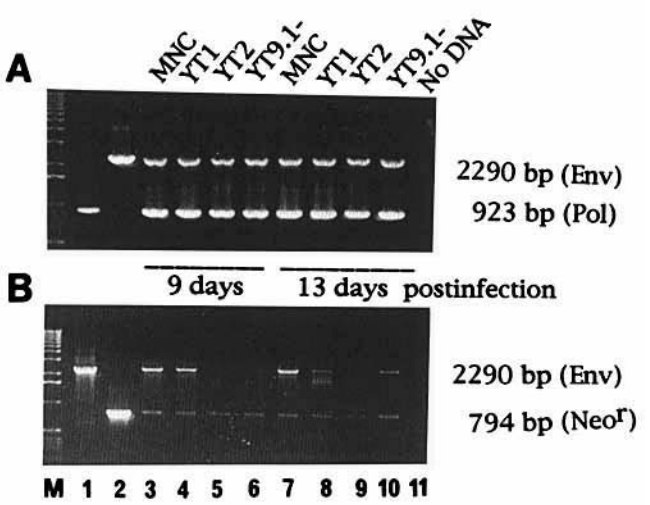


Fig. 5. PCR analysis of transduced CEM174 cells. Total cellular DNA was extracted from infected cells (0.01 moi). Half μg and one ng of total DNA was co-amplified with *env* and *pol* **(A)** and *env* and *Neo*^{r} (B), respectively. One-hundred pg pSIVSpE3′ (**A,** lane 2 and B, lane 1), pSIVSpSp5′ (A, lane 1), and pZipNeoSV(X) (**B,** lane 2) were the controls.

degrees of success. A complex stoichiometry is involved in this process. The stability of the structure, molar ratio of antisense and target sequence, and the size of antisense RNA all play a significant role in effective inhibition. For any gene therapy approach to be effective in the inhibition of viral replication, a high copy number or an optimal size of antisense RNA should be present in the protected cells. The goal for gene therapy of AIDS is to introduce an antiviral gene into hematopoietic stem cells. Cell lineages derived from transduced stem cells eventually will all carry antiviral genes. Introduction of a high copy number of antiviral genes into stem cells and their progeny would be difficult to achieve in this case. Therefore, our research focus is to determine the optimal size of antisense RNA for inhibition of viral expression or replication.

In this study, a single-target model was used and clearly demonstrated that the size of antisense RNA played a significant role in protection. Clonal variation and asynchrony of expression may contribute to the variation of antisense RNA levels in transduced cells. In order to minimize variations, the pool of antisense transduced cells were cultured in exponential growth condition in the assay. From the Northern analysis, no degraded RNA was detected that indicated the stability of the antisense RNAs. However, the secondary structure of antisense RNA also may contribute to the effective inhibition. The 4.5 Kb antisense RNA did not show an inhibitory effect in our assay. This phenomenon could be due to nonspecific binding of unrelated cellular sequences and/or different cellular compartmentation of antisense and viral RNAs. It is worth noting that the multiple target sequence, i.e., envelope region, still could be the better target for antisense mediated inhibition in clinical applications. In that case, both full-length and spliced viral sequences could be inhibited by antisense RNA.

The results also demonstrated that the infectious dose plays an important role in inhibition, which is consistent with published data in HIV system [Sczakiel et., 1992]. However, the calculation of infectious dose in the present study was more accurate than in previous investigations because the same cell line was used for growing the virus, titering it, and for challenge assay. At all these multiplicities of infection (0.01–1.0), any individual cell would have been infected only with one infectious virus particles, and thus only the proportion of infected to uninfected cells would have varied. Because effective inhibition only occurred at a low M.O.I. (0.01), it is suggested that the antisense RNA may block the spread of viral infection from 1% of infected cells to the predominant population of uninfected cells. Therefore, it is postulated that antisense RNAs may function better in the early stage of viral infection when viral load is low.

Many other molecular targeting approaches are being explored to develop effective treatment to slow down viral replication, such as transdominant negative mutants, RNA decoys, and ribozymes [Liu et al., 1994; Lori et al., 1994; Yamada et al., 1994; Yu et al., 1994]. Large antisense RNA becomes an attractive approach for anti-HIV therapy because of its properties of non-immunogeniecity, viral specificity, and multiple targets. There is a lack of a standard assay to compare the effectiveness among different approaches; however, the assay system used in this report is closer to natural infection, at least, in the early stage of infection in patients. Many points discussed in this report should be taken into consideration when other molecular targeting approaches are to be evaluated before clinical trials.

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